Biological Production of Methanol from Methane

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Cultures of methanotrophs have been isolated that convert methane into methanol. Biocatalytic conversion offers the advantages of good thermal efficiency and low capital cost, since ordinary temperatures and pressures are employed. High product yield in a single-step reaction and simplified purification technology are possible, since methanol is the only product.

These unusual bacteria usually metabolize methane completely to CO_2, with methanol as an intracellular intermediate. Therefore, methanol production requires manipulation of the ordinary enzymatic reactions by regulation of the electron transport and environmental conditions to favor the methane monooxygenase pathway. This paper presents preliminary results of the culture isolation techniques and procedures to manipulate the cultures to produce methanol. These procedures have been successfully demonstrated with two isolates producing up to 1 g/L methanol extracellularly.

INTRODUCTION

Methanol is a major raw material for petrochemical production and is currently under consideration as a liquid fuel. Methanol is produced catalytically by the reaction of hydrogen and carbon monoxide at high pressure (300 psia) and moderate temperature (Strelzoff, 1970). H_2 and CO are obtained from methane by reforming with steam to yield synthesis gas. A second step, involving a water-gas shift reaction, is used to increase the H_2/CO ratio. The reforming step is generally carried out at 800-1000°C and 300 psig, whereas the water gas shift reaction utilizes metal oxide catalysts at 400-500°C and 300 psig (Shah and Stillman, 1970; and Shreve, 1967). These severe conditions result in high capital and operating costs and poor thermal efficiencies. Simpler, more efficient processes are necessary.

Natural gas demand and production declined to less than 17 trillion cu. ft. last year, with reserves dropping below eight years (Oil Gas J., 1986a; Beck, 1987). By 1990, gas imports are expected to be up 300 percent over present levels, despite a continuing decline in demand (Oil Gas J., 1986b). While flaring of natural gas has been substantially reduced in recent years, the U.S. presently wastes about 10×10^{10} cu. ft. annually (Hillard, 1980), or the equivalent of 1.4 billion gallons of methanol (40 percent of our liquid fuel requirement). Also, in many areas, gas wells remain shut-in because potential gas production is too remote or two dilute to justify pipelines and transportation. If simple conversion technology were available to produce liquid fuels on-site, flared and remote gas could be utilized.

Catalytic conversion of coal synthesis gas, using Fischer-Tropsch reactions, has been found to produce methanol and higher alcohols with sustained catalyst activity (Klier *et al.*, 1986; Dombek, 1986). By-products of the Fischer-Tropsch reactions include light hydrocarbon gases, predominantly methane. Methane is also a by-product from some gasification

processes (Mills, 1982). Methane is very stable in the subsequent processing steps to produce liquid fuels. Therefore, a technology or catalyst for converting methane directly into liquid fuels would substantially enhance the efficiency and yields of these processes.

A simple and efficient process for producing methanol from methane would save significant quantities of energy in industrial processes. Also, technology for conversion of small volumes or dilute mixtures of methane would enable production from remote gas and oil wells, saving wasted energy and reducing imports of crude oil. Furthermore, this technology would enhance the application of coal conversion technologies. Methanol production from methane is a mature technology and substantial advances or breakthroughs in catalytic processes are not likely. Therefore, innovative approaches to this problem are necessary.

Biological Conversion of Methane

Of the many biological species and microbiological reactions possible, only the methanotrophs are capable of converting methane. The usual sequence of methane metabolism proceeds to cell biomass and CO_2 with methanol as an intracellular intermediate. The interruption of the enzymatic reactions, by manipulation of the environmental conditions or mutation, could result in a culture that produces an excess of methanol that accumulates extracellularly.

Such a biocatalytic process has substantial economic potential for application to Fischer-Tropsch products, coal synthesis gas and natural gas conversion. Methane, in the gas stream following the Fischer-Tropsch synthesis, would be passed through the biological reactor for conversion to methanol. Similarly, methane in synthesis gas could be converted to methanol prior to or following Fischer-Tropsch synthesis. The conversion would take place in a single step at ordinary temperatures and at atmospheric or elevated pressures, if desirable. No products, other than methanol, are produced by these cultures. Complete methane conversion, with near stoichiometric yields, should be possible. Methanol recovery from the fermentation media could be accomplished by stripping or liquid extraction (extractive fermentation).

Purpose

The purpose of this paper is to present the results from preliminary laboratory experiments aimed at isolating a methanotroph culture that is capable of accumulating methanol as a product. Two isolates have been obtained that are capable of producing up to 1 g/L extracellularly.

MICROBIOLOGY OF METHYLOTROPHS

The biological conversion of methane to methanol by the reaction:

$$CH_4 + 1/2\ O_2 \rightarrow CH_3OH \qquad (1)$$

is carried out by a very specialized group of organisms that are also able to utilize methanol, methylamine or formate (in addition to methane) as sole carbon and energy sources. This class of organisms is called methylotrophs. Organisms that utilize primarily methane are called methanotrophs.

Organisms such as Methylococcus capsulatus and Methylomonas sp. are considered obligate methylotrophs and cannot grow on carbon sources other than methane, methanol, or formate. Energy is derived from the oxidation of these compounds to carbon dioxide, and most of the carbon is obtained from the fixation of formaldehyde by condensation with a pentose phosphate. Microorganisms that cannot utilize methane but can utilize methanol, methylamine and formate (e.g. Hyphomicrobium and a few Pseudomonas species) are generally also considered to be methylotrophs. Two types of methylotrophs have been established on the basis of the type of complex membranous organelles. Type I exhibits a system of paired membranes running throughout the cell or aggregated at its periphery. Type II exhibits a series of bundles composed of disc-shaped membrane vesicles distributed throughout the cell.

Type I methylotrophs utilize the ribulose monophosphate pathway for the assimilation of formaldehyde and have an incomplete tricarboxylic acid cycle. These organisms lack alpha-ketoglutarate dehydrogenase (Patel, 1984). Type II methylotrophs utilize the serine pathway for the assimilation of formaldehyde and have a complete tricarboxylic acid cycle (Large, 1983).

The methane molecule can only be attacked by a substitution mechanism. It was shown in 1970 that growth on methane is accompanied by the incorporation of an oxygen atom from gaseous oxygen into the molecule to give methanol, as was shown in Equation (1). Methane is actually oxidized finally to CO_2, with methanol, formaldehyde, and formate formed as intermediates (Anthony, 1982; and Higgins *et al.*, 1981a):

$$CH_4 + O_2 + 2H^+ \rightarrow H_2O + CH_3OH \rightarrow 2H^+ + HCHO \xrightarrow{1/2O_2} 2H^+ + HCOO^- H^+ \rightarrow CO_2 + 2H^+ \quad (2)$$

This series of reactions occurs intracellularly and no methanol is produced extracellularly. In order for the methanotroph to produce methanol, conditions for the subsequent enzymatic reactions must be made unfavorable. The enzyme, methane monooxygenase, catalyzes the reaction:

$$CH_4 + O_2 + H^+ + NADH \rightarrow NAD^+ + CH_3OH + H_2O \quad (3)$$

The enzyme occurs in both soluble and particulate form. The physical location of the enzyme as a cytoplasmic or extracytoplasmic enzyme has not been determined. Noting that NADH is a substrate, it is generally assumed that the reactions are cytoplasmic. This assumption is also consistent with the fact that they are proton utilizing (Hooper and DiSpirito, 1985). Reducing equivalents from the formaldehyde, formate, and perhaps methanol dehydrogenase reactions are utilized in methane monooxygenase, in the reduction of NAD^+ for biosynthetic reactions, or in electron transport leading to ATP synthesis.

Although the prosthetic group of methanol dehydrogenase is a novel quinone coenzyme (pyrroloquinoline quinone) the enzyme utilizes a soluble cytochrome c as an electron acceptor and is therefore a proton-yielding dehydrogenase (Beardmore-Gray *et al.*, 1983). Localization studies have shown that methanol dehydrogenase (Alefounder and Ferguson, 1981; Burton *et al.*, 1983; and Kasprzak and Steenkamp, 1983; 1984) and the electron acceptors cytochromes C_L, and possibly cytochrome C_H (Beardmore-Gray *et al.*, 1983; Burton *et al.*, 1983;

Jones *et al.*, 1982; and Quilter and Jones, 1984), are in the periplasm (Alefounder and Ferguson, 1981; and Kasprzak and Steenkamp, 1983). Thus, methanol oxidation clearly fits the generalization described in Equation (3) by Hooper and DiSpirito (1985).

Formaldehyde and formate dehydrogenase occur in forms which use either NAD^+ or dyes as electron acceptors (Johnson and Quayle, 1964; and Marison and Wood, 1980). Substrate oxidation is proton yielding and could logically be periplasmic:

$$H_2O + HCHO \rightarrow 2H^+ + 2e^- + HCOOH \quad (4)$$

and

$$HCOOH \rightarrow 2H^+ + 2e^- \; CO_2 \quad (5)$$

Depositing cross-membrane translocation of electrons to the proton-utilizing reduction of NAD^+:

$$H^+ + 2e^- + NAD^+ \rightarrow NADH, \quad (6)$$

the action of a dehydrogenase located in the periplasm would generate a proton gradient in both reactions. This seems not to be the case with the NAD-linked formaldehyde (Kasprzak and Steenkamp, 1983) and formate (Jones *et al.*, 1982) dehydrogenases; since they are soluble and use NAD, they are probably cytoplasmic. The topological location of the dye-linked enzymes is unknown.

In summary, in the methylotrophs, the proton-utilizing methane monooxygenase may be cytoplasmic and the energy-linked proton-yielding oxidation of methanol is periplasmic. Thus, CH_3OH functions as a transmembrane hydrogen transporter. In contrast, NAD-linked oxidations of formaldehyde and formate, which produce reductant for methane monooxygenase, biosynthesis, or electron transport, leading to ATP synthesis and CO_2 for carbon assimilation, are apparently cytoplasmic. Therefore, mechanisms for control of these separate enzyme systems to allow methanol production is feasible and likely. Furthermore, the potential to control electron flow and block specific enzyme sites with inhibitors to increase methanol yields is substantial.

THE PRODUCTION OF CHEMICALS USING METHYLOTROPHS

It is possible to biologically convert methane to methanol using whole cell systems or cell-free enzyme systems. Cell-free systems require an external source of reducing energy supplied by NADH for substrate oxidation. The methylotrophic enzyme which is of greatest interest is methane-monooxygenase which catalyzes the reaction:

$$CH_4 + O_2 + H^+ + NADH \rightarrow NAD^+ + CH_3OH + H_2O \quad (3)$$

This enzyme, although of fundamental importance for bacterial growth on methane, may become industrially important for quite a different reason. Since it is such a non-specific enzyme, it will oxidize a wide number of compounds in addition to methane (Large, 1983), some of which cannot be transformed by traditional industrial chemical methods.

Methane monooxygenase enzyme involved in catalyzing the hydroxylation of methane also catalyzes the oxygenation of various hydrocarbons and cyclic, alicyclic and aromatic compounds (Colby and Dalton, 1976; Colby *et al.*, 1977; Dalton, 1980; Dalton and Colby, 1982; Higgins *et al.*, 1981b; and Patel *et al.*, 1979). Thus, it is possible to convert methane to methanol or other short-chain hydrocarbons to their alcohols using whole cell systems. Patel (1984) reported that in using the soluble enzyme, methane monooxygenase, extracted from Methylobacterium sp. strain CRL-26, he was able to oxidize methane to methanol, ethane to ethanol, propane to 1-propanol and 2-propanol, butane to 1-butanol and 2-butanol and pentane to 1-pentanol and 2-pentanol, etc.

However, unlike simple hydrolases or oxidases, mixed-function oxidases, such as methane monooxygenase, need reduced nicontinamide nucleotides in addition to oxygen in order to function. Cell free systems, such as an immobilized enzyme system, are thus considered costly and impractical to use for the conversion of methane to methanol.

As an alternative to cell free systems, it is also possible to convert methane to methanol using whole cell systems, which supply their own reducing equivalents by substrate oxidation. Methane-utilizing organisms, grown on methane or methanol, have the ability to oxidize and transform a variety of non-growth substrates to commercially useful chemicals. This is due to the broad specificity of enzymes involved in the oxidation of methane to carbon dioxide. The isolation of such organisms, particularly those that might produce methanol from methane, would have broad application.

RESULTS AND DISCUSSION

CULTURE ISOLATION

Experimental studies were carried out in an effort to isolate methanotrophs from anaerobic digester sludge. This source of inoculum was chosen since the sludge contains significant quantities of methane in the liquid phase, and should, therefore, contain methanotrophs capable of using the methane.

Digester sludge was inoculated into a mineral salts medium, shown in Table 1, using a 10 percent by volume inoculum. As noted, the medium was essentially a salts medium, but also contained vitamins found essential for methanogen growth. The gas phase above the liquid media was maintained at 1 atm, and contained 20 volume percent methane and 80 percent air.

A mixed culture developed from this initial seeding was enriched by successive transfer to new media and gas every 72 hours. The enrichment procedure lasted a total of approximately 8 weeks. The enrichment was then

Table 1

Medium for Methylotroph Isolation

	g/L
KNO_3	2.5
Na_2HPO_4	0.21
KH_2PO_4	0.29
$MgSO_4 \cdot 7H_2O$	0.20
$FeSO_4 \cdot 7H_2O$	0.001
Trace elements[a]	1ml/L
Vitamins[b]	10ml/L

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a The trace elements stock solution, shown below, were diluted 1000 times prior to use.

	mg/L
$CuSO_4$	50
H_3BO_3	10
$MnSO_4 \cdot 4H_2O$	10
$ZnSO_4 \cdot 7H_2O$	70
MoO_3	10

b A methanogen minimal medium, shown below, was diluted 100 times prior to use.

Biotin	2
Folic acid	2
Pyridoxine HCl	10
Thiamine HCl	5
Riboflavin	5
Nicotinic Acid	5
Ca-pantothenate	5
Vitamin B_{12}	0.1
p-amino benzoic acid	5
Thioctic acid	5

streaked onto agar plates utilizing the same media as in Table 1, and incubated under an atmosphere of 20 percent methane, 80 percent air until colonies appeared.

Individual colonies were picked, inoculated into fresh liquid media, and examined for purity. Two of the isolates that were obtained were obligate methanotrophs, and thus were selected for further testing.

CONVERSION OF METHANE TO METHANOL

Utilizing the obligate methanotrophs isolated from digester sludge, experiments were initiated to determine the feasibility of producing methanol from methane. Stoppered 250-ml Erlenmmeyer flasks were used as batch reactors. The liquid media utilized in the experiments was identical to the media of Table 1.

To facilitate mass transfer of methane from the gas phase to the liquid phase for reaction, gentle agitation (approximately 150 rpm) was employed, using a shaker incubator. The organisms were again grown under an atmosphere of 20 percent methane and 80 percent air at one atmosphere total pressure. Two obligate methanotroph isolates were compared in the study, along with the enrichment culture obtained after successive transfer from the digester sludge.

After obtaining growth in the reactors, the gas phase was switched to 100 percent methane in place of the 20:80 methane/air mixture. This substitution was made in order to prevent complete oxidation of methane to CO_2 as was shown in Equation (2). Incubation with gentle agitation occurred for 24 hours.

The results of these preliminary studies is shown in Table 2. Liquid phase analysis for methanol was performed using gas-solid chromatography. As noted, the two pure culture methanotrophs showed an accumulation of methanol, producing 0.5 and 1.0 g/L methanol in 24 hours. These levels of methanol production in 24 hours indicate the potential for good reaction rates in continuous culture. The enrichment, which contained methanotrophs and

Table 2

Biological Conversion of Methane to Methanol
(Preliminary Studies)

	Methanol Produced After 24 hours (g/L)
Isolate #1	0.5
Isolate #2	1.0
Enrichment	0

methanol-utilizing methylotrophs, showed no accumulation of methanol. The differences in the methanol production by the methanotrophs can be contributed to sensitivity to the presence of methanol, activity of the culture, and the ability of the cultures to utilize methanol as a substrate.

The results of these preliminary experiments are quite encouraging and demonstrate that the organisms are capable of producing methanol extracellularly at fast rates. Low cell densities and gentle agitation were used and, consequently, low methanol concentrations were obtained in the short reaction time. A measurement of the cell density in the flask showed a very low cell concentration of 0.3 g/L on a dry weight basis. A higher cell mass concentration (analogous to a higher catalyst concentration) would increase reaction rate to yield more product in the 24 hour period.

The experimental conditions can undoubtedly be improved to maximize methanol yields. Also reaction rates would be increased by providing better mass transfer of methane and oxygen from the bulk gas phase to the organisms in the liquid phase. The total pressure in the experiments was only 1 atmosphere. Higher pressures would be expected to significantly enhance reaction rate. Significantly higher methanol concentrations should be possible under improved conditions of cell density, reaction conditions and system design.

REFERENCES

Alefounder, P. R. and S. J. Ferguson. 1981. A periplasmic location for methanol dehydrogenase from Paracoccus denitrificans, implications for proton pumping by cytochrome aa_3. Biochem. Biohys. Res. Commun. 98: 778-784.

Anthony, C. 1982. The Biochemistry of Methylotrophs. Academic Press, Inc., New York.

Beardmore-Gray, M., D. T. O'Keefe, and C. Anthony. 1983. The methanol: cytochrome C. oxidoreductase activity of methylotrophs. J. Gen. Microbiol. 129: 923-933.

Beck, R. J., Oil and Gas J., 42 (Jan 26, 1987).

Burton, S. M., D. Byrom, M. Carrer, G.D.D. Jones and C. W. Jones. 1983. The oxidation of methylated amines by the methylotrophic bacterium Methylophilus methylotrophus. FEMS Microbiol. Lett. 17: 185-190

Colby, J. and H. Dalton. 1976. Some properties of a soluble methane monooxygenase from Methylococcus capsulatus strain Bath. Biochem. J. 157: 495-497.

Colby, J., D. I. Stirling and H. Dalton. 1977. The soluble methane monooxygenas of Methylococcus capsulatus (Bath.). Its ability to oxygenate n-alkanes, n-alkenes, ethers and alicyclic, aromatic and heterocyclic compounds. Biochem. J. 165: 395-402.

Dalton, H. 1980. Oxidation of hydrocarbons by methane monooxygenase from a variety of microbes. Adv. Appl. Microbiol. 26:71.87.

Dalton, H., and J. Colby. 1982. Methane monooxygenase: an iron-sulfur flavoprotein comples. p. 763-767. In V. Massey and C. H. Williams (ed.) Flavins and Flavoprotein. Elsevier/North-Holland Pub. Co., N.Y.

Dombek, D. B. 1986. Optimum Fuel Alcohol Mixtures from Syngas Oxygenate Synthesis, Proceedings Indirect Liquefaction Review, PETC.

Higgins, I. J., D. Best, R. C. Hammond, and D. Scott. 1981a. Methane-oxidizing microorganisms. Microbiol. Rev. 45:556-590.

Higgins, I. J., D. Best and D. Scott. 1981b. Hydrocarbon oxidation by Methylosinas trichosparium: Metabolic implication of the lack of substrate specificity of methane monooxygenase. p. 11-20. In H. Dalton (ed.) Microbial Growth on C_1 Compounds. Hayden Publishing Co., London.

Hillard, J. H., Natural Gas, Kirk-Othmer Encyl. of Chem. Tech., 11, 630, Wiley (1980).

Hooper, A. B. and A. A. DiSpirito. 1985. In bacteria which grow on simple reductants, generation of a proton gradient involves extracytoplasmic oxidation of substrate. Microbiol. Rev. 49: 140-157.

Johnson, P. A., and J. R. Quayle. 1964. Microbial growth on C_1 compounds. Oxidation of methanol, formaldehyde and formate by methane-grown Pseudomonas AMI. Biochem. J. 93: 281-290.

Jones, C. W., S. A. Kingsbury, and M. J. Dawson. 1982. The partial resolution and dye mediated reconstitution of methanol oxidase activity in Methylophilus methylotrophus. FEMS Microbiol Lett. 13: 195-200

Kasprzak, A. A. and D. J. Steenkamp. 1983. Localization of the major dehydrogenases in two methylotrophs by radiochemical labeling. J. Bacteriol. 156:348-353.

Klier, K., K. J. Smith and J. G. Nunan. 1986. Direct Synthesis of Methanol ls, Oxygenate Synthesis - F-T Products, Proceedings Indirect Liquefaction Review, PETC.

Large, P. J. 1983. Methylotrophy and methanogenesis. American Society for Microbiology, Washington, D.C.

Marison, I. W., and A. H. Wood. 1980. Partial purification and characrization of a dye-linked formaldehyde dehydrogenase from Hyphomicrobium X. J. Gen. Microbiol. 117:305-313.

Mills, G. A. Synfuels from Coal Progress in USA. 1982. Energy Progress, (57).

Patel, R. N. 1984. Methane Monooxygenase from Methylobacterium sp. ain CRl-26, in Microbial Growth on C_1 Compounds. (R. L. Crawford and R. S. Hanson eds.) pp 83-90.

Patel, R. N., C. T. Hou, A. I. Laskin, A. Felix, and P. Derelanko. 1979. Microbial oxidation of gaseous hydrocarbons: hydroxylation of n-alkanes and epoxidation of n-alkenes by cell-free particulate fractions of methane utilizing bacteria. J. Bacteriol. 139:675-679

Quilter, J. A. and C. W. Jones. 1984. The organization of methanol dehydrogenase and C-type cytochromes on the respiratory membrane of Methylophilus methylotrophus. FEBS Lett. 174:167-172.

Shah, M. and J. Stillman. 1970. Ind. Engr. Chem. Proc. Des. and Dev. 62 (12) p. 59.

Shreve, R. N. 1967. Chemical Process Industries, McGraw-Hill, New York.

Strelzoff, S., Chem. Engr. Symp. Ser., 66, 98 (1970).

_____, Oil & Gas J., 29 (Oct. 6, 1986a).

_____, Oil & Gas J., 36 (Sept. 29, 1986b).